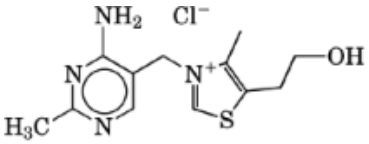
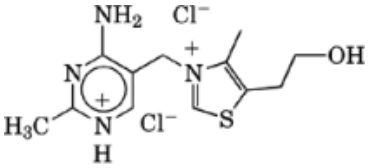
Kirk-Othmer Encyclopedia of Chemical Technology.

THIAMINE (B_1)

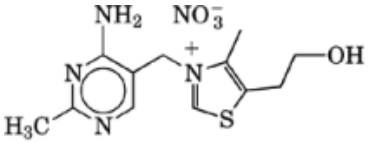
Thiamine [59-43-8] is the official IUPAC-IUB name for 3-(4-amino-2-methyl-5pyrimidinyl) methyl-5-(-2-hydroxyethyl)-4-methylthiazolium chloride, $C_{12}H_{17}N_4OSCl$ **(1)**. The names thiamine, thiamin (used in many official and commercial documents), aneurine, and the older term vitamin B_1 are also casually used. These names usually refer to the chloride hydrochloride [67-03-8] **(2)**, the common biological and commercial form.



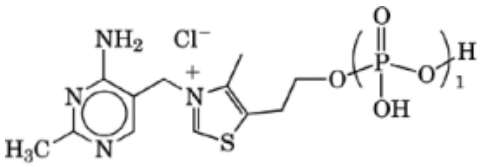
(**1**)



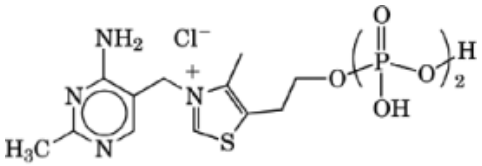
(**2**)



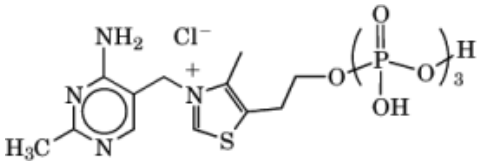
(**3**)



(**4**)



(5)



Thiamine is found in varying, low levels as its salts and phosphate esters in the tissues of practically all life forms, where its pyrophosphate [154-87-0] **(5)**, known as cocarboxylase, plays essential roles in carbohydrate metabolism. Plants and most microorganisms biosynthesize thiamine, but animals are incapable of doing so, require it in their diets in amounts varying with their carbohydrate use, and excrete the excess. Thiamine content of foods varies and can be partially lost during storage or processing, as it is one of the less stable of the vitamins. Thiamine deficiencies can lead to a range of effects, from the malaise and fatigue of a lesser deficiency to the serious disorders of gross deficiency observed for many animal species, such as beriberi, where weight loss, heart disease, and serious neurological degeneration can lead to death. As preventive measures, thiamine is used to enrich foods and feeds and as an ingredient of dietary supplements. Thiamine is also used therapeutically for treatment of specific deficiency conditions. Thiamine was one of the first of the vitamins to be manufactured for commerce. The common commercially available forms of thiamine, the chloride hydrochloride **(2)** and the mononitrate [532-43-4] **(3)**, are manufactured by chemical processes operated at fine chemical scales. At present, natural sources and bioprocesses are not cost-competitive for bulk production.

The history of thiamine is linked with the disease beriberi, which once caused a great toll of suffering and death in parts of the world where milled or polished rice is a staple of the diet (1). Beriberi was recognized in China as early as 2600 BC, but the first cure was only demonstrated in 1885. Takaki, then surgeon general of the Japanese navy, eradicated beriberi in the fleet by adding more protein to the sailors' diet of mainly refined rice to achieve a more Western-style diet. By 1901 the Dutch physicians Eijkman and Grijn showed hens kept on a diet of de-husked rice developed a disease similar to human beriberi which could be reversed or prevented by adding rice bran and other nitrogenous materials to their feed. They suggested beriberi was caused not by pathogens or toxins but by the lack of a vitally important food constituent, which was a radical idea at the time. They also showed the preventive factor was leached by water and destroyed by heat. Searches for a specific water-soluble nitrogenous substance led the English chemist Funk in 1911 to coin the term vitamine (vital amine, an amine essential for life), popularizing the concept of deficiency diseases. In 1926, a small, pure crystalline sample of thiamine hydrochloride was painstakingly first isolated from rice bran extracts by Dutch chemists Jansen and Donath. After eight years of effort, in 1932 the German chemist Windaus and his co-workers obtained pure thiamine from yeast extracts and established the correct empirical formula. In 1935–1936, following similarly prolonged isolation efforts, the American chemists Williams and Cline and German chemist Grewe independently proposed the correct structure based on degradation studies. The name thiamine was first used by Williams. Shortly thereafter, the Williams group confirmed the structure by rational synthesis (2), followed closely by two different syntheses by other workers (3–5). Whereas in 1933 Williams had succeeded in isolating only gram quantities from metric tons of rice polishings, a highly enriched source, in 1937 chemists at Merck and Hoffmann-La Roche developed a production level of about 100 kg within a

year based on two different, relatively involved chemical syntheses. Demand and production levels have risen steeply since then to their present estimated level of about 3300 t/yr worldwide.

1. Physical and Chemical Properties

1.1. Salt Formation

As a weakly basic pyrimidine and a thiazolium cation, thiamine forms both mono- and dipositive salts, eg, the two commercial forms.

Thiamine chloride hydrochloride [67-03-8], (thiamine hydrochloride) $C_{12}H_{18}N_4OSCl_2$ **(2)**, crystallizes as colorless monoclinic needles, mp 248–250°C (with decomposition), which in bulk appear white and have an approximate density of 0.4 kg/L. Several polymorphic crystal forms have been reported. The salt has a characteristic thiazole meat-like odor and a slightly bitter taste. On exposure to air of average humidity, the hydrochloride **(2)** can adsorb up to one mole of water (more typically slightly less, to about 4% by weight), which may be removed by heating to 100°C or by vacuum drying. It is very soluble in water (over 1 kg/L at 25°C), soluble in glycerol (0.056 kg/L), propylene glycol, and methanol, sparingly soluble in 95% ethanol (0.01 kg/L), and practically insoluble in less polar organic solvents. In 1–5% solutions in water it shows a pH of 3–3.5 (6, 7).

Thiamine mononitrate [532-43-4] **(3)**, $C_{12}H_{17}N_5O_4S$, is an apparently white, colorless crystalline solid with a typical odor, melting point of ca 196–200°C (dec.) and an approximate bulk density of 0.5 kg/L. It is much less soluble in water than the hydrochloride (0.027 kg/L at 25°C, 0.030 kg/L at 100°C) and practically nonhygroscopic. Dilute solutions in water show a pH of 6.5–7 (6, 8).

Numerous other salts have been reported in the literature, including some which are insoluble in water.

1.2. Physical Chemical Characterization

Thiamine, its derivatives, and its degradation products have been fully characterized by spectroscopic methods (9, 10). The ultraviolet spectrum of thiamine shows pH-dependent maxima (11). ^1H, ^{13}C, and ^{15}N nuclear magnetic resonance spectra show protonation occurs at the 1-nitrogen, and not the 4-amino position (12–14). The ^1H spectrum in D_2O shows no resonance for the thiazole 2-hydrogen, as this is acidic and readily exchanged via formation of the thiazole ylid 1 an important intermediate in the biochemical functions of thiamine. Recent work has revised the pK_a values for the two ionization reactions to 4.8 and 18 respectively (9, 10, 15). The mass spectrum of thiamine hydrochloride shows no molecular ion under standard electron impact ionization conditions, but fast atom bombardment and chemical ionization allow observation of both an intense peak for the parent cation and its major fragmentation ion, the pyrimidinylmethyl cation (16).

1.3. Reactions and Stability

Thiamine hydrochloride is stable as a solid if kept dry. Heating to 100°C for 24 h does not diminish its potency. In solution, its stability depends heavily on conditions of pH, temperature, and oxygen. Aqueous solutions below pH 5 are stable to oxygen, heating, and even autoclaving (11). Heating in water to 140°C under pressure gives decomposition to 4-amino-5-hydroxymethyl-2-methyl-pyrimidine [73-67-6] **(7)** and 4-methyl-5-(2-hydroxyethyl)thiazole [137-00-8] **(8)** (17). Heating with 20% hydrochloric acid hydrolyzes the 4-amino group to yield oxythiamine [582-36-5] **(9)**, an antagonist of thiamine (18). Above pH 5, aqueous thiamine is destroyed relatively rapidly by boiling. At pH 7 destruction occurs even at room temperature. Concentrated solutions of thiamine in alcohol when neutralized degrade rapidly at room temperature to liberate thiazole **(8)** and form oligomers of the pyrimidine where the 5-methylene is linked to N-1 of another pyrimidine unit (10, 14).

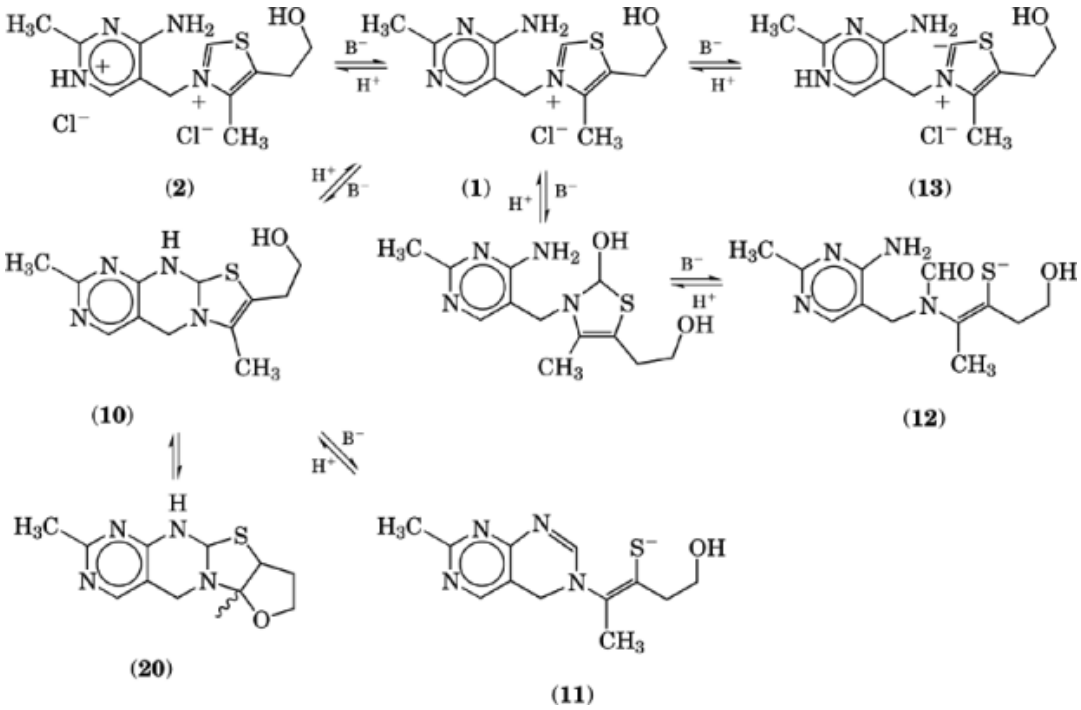
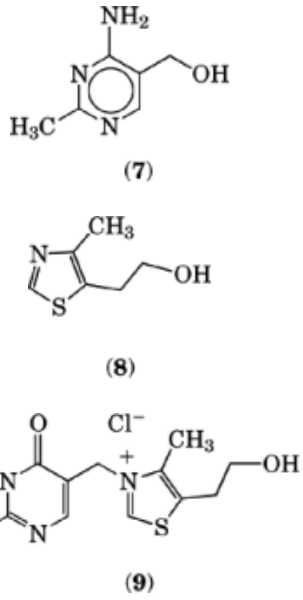


Fig. 1. Structural changes of thiamine with pH.



At pH 8 or above, even at room temperature, water solutions of thiamine turn yellow, then fade as a series of reactions ensues (9, 10, 19). Basification allows intramolecular attack of the 4-amino function on the thiazole ring to generate dihydrothiochrome [80483-97-2] 1, which eliminates thiolate to generate the salt of the

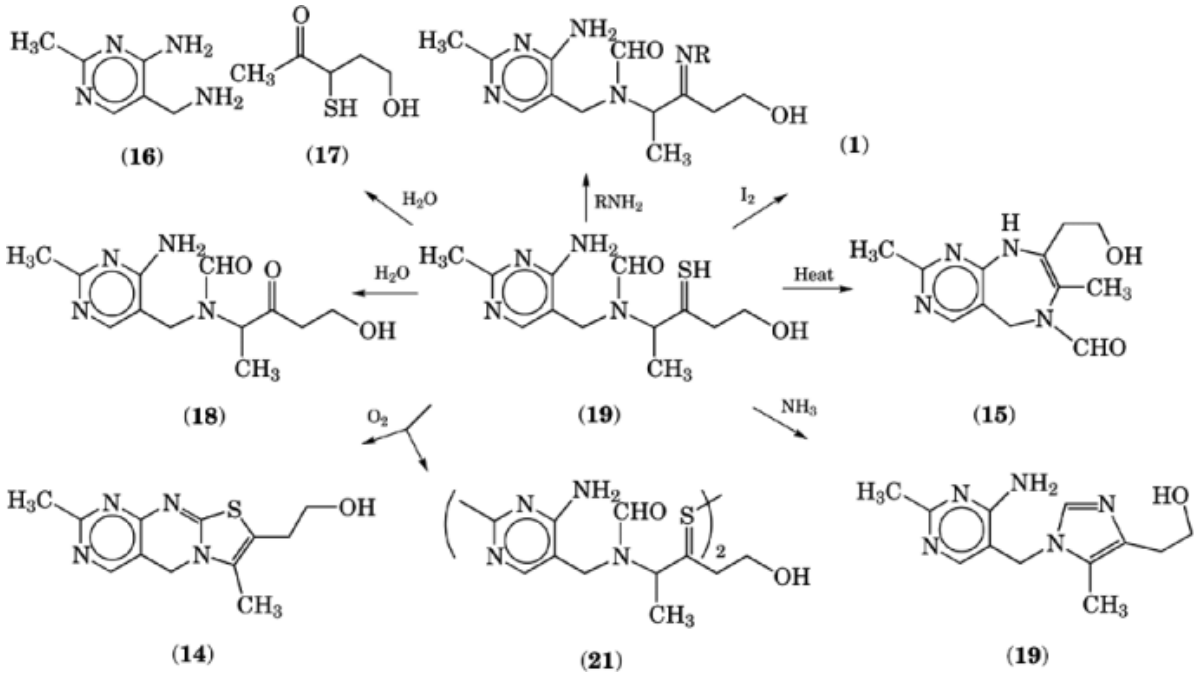


Fig. 2. Reactions of the thiol form (**12**).

yellow form, 5,6-dihydropyrimido(4,5*d*)pyrimidine [84825-03-6] 1, as a kinetic product (9, 10, 19) (Fig. 1). The sequence is reversible. More slowly, under the same conditions, a competing hydrolysis of the thiazolium ring via the pseudobase generates another product, known as thiamine thiol or the thiol form [554-45-0] 1 as the thermodynamic product. Also present in the system is a small amount of thiamine ylid [84812-92-0] 1, formed by deprotonation of C-2. The products dihydrothiochrome 1, yellow form 1, and ylid 1 undergo significant and useful chemistry.

The yellow form 1 on acidification is converted to the more stable thiol form 1. On oxidation, typically with alkaline ferricyanide, yellow form 1 is irreversibly converted to thiochrome [299-35-4] 2, a yellow crystalline compound found naturally in yeast but with no thiamine activity. In solution, thiochrome exhibits an intense blue fluorescence, a property used for the quantitative determination of thiamine.

The thiol form 1 undergoes reactions mainly via its *N*-formyl or ene–thiol groups. Heating an aqueous solution of the thiol form (Fig. 2) effects hydrolysis to the diamine 4-amino-5-aminomethyl-2-methyl-pyrimidine [95-02-3] 2, 5-hydroxy-3-mercaptopentan-2-one [15678-01-0] 2, and formic acid (20). Neutralization of a solution of the thiolate anion with carbon dioxide gives a fat-soluble basic material [21682-72-4, 35922-43-1] 2, presumably via dihydrothiochrome 1 (21). Acylation of the thiolate occurs on both sulfur and oxygen to give mono- or diacyl thiamines, some of which are interesting fat-soluble depot forms of thiamine.

The thiol form 1 is susceptible to oxidation (see Fig. 2). Iodine treatment regenerates thiamine in good yield. Heating an aqueous solution at pH 8 in air gives rise to thiamine disulfide [67-16-3] 2, thiochrome 2, and other products (22). The disulfide is readily reduced to thiamine *in vivo* and is as biologically active. Other mixed disulfides, of interest as fat-soluble forms, are formed from thiamine, possibly via oxidative coupling to the thiol form 1.

Whereas a claim of isolation of the thiamine ylid 1 has been the subject of controversy (15, 23), kinetic and product studies and molecular orbital calculations support the formation and reactivity of a thiamine

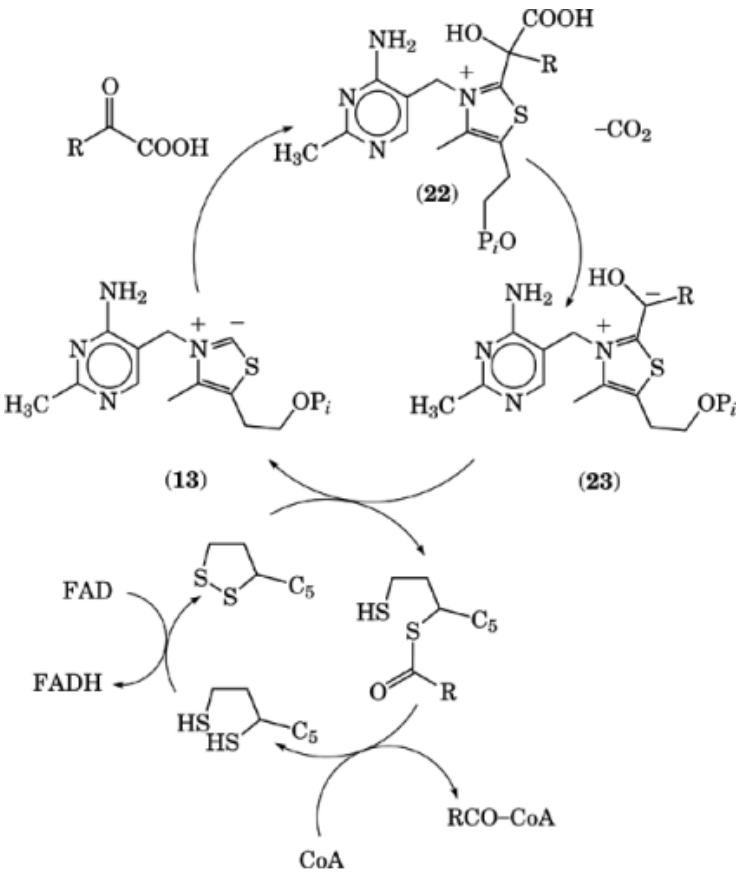


Fig. 3. Oxidative decarboxylation of alpha-ketoacids.

ylid as an unstable intermediate (15, 24–26). Ylid 1 is accepted as an intermediate in explanations of the enzymatic and nonenzymatic reactions of thiamine. Among these reactions are the enzymatic oxidative decarboxylation of pyruvic and 2-ketoglutaric acids, the formation of acetoin, the reversible alpha-ketol transfer reactions catalyzed by transketolase, and the nonenzymatic acyloin condensation. According to the thiazolium ylid mechanism, ylid 1 reacts reversibly with carbonyl reagents, facilitating aldol–retroaldol and decarboxylation reactions (Fig. 3), via the acyl anion equivalent thiazolium ylids 3, the so-called active aldehydes, which are the key intermediates (27). In the specific case of oxidative decarboxylation of pyruvic acid, for example, ylid 3 transfers the acyl group to lipoic acid. Other reactions involving thiamine similarly involve nucleophilic additions of a thiazolium ylid to a suitable acceptor. Syntheses of a thiamine–pyruvate adduct 3 and a protonated form of its decarboxylation product 3 (28), as well as model studies with thiazolium ions (29), further support the thiazolium ylid mechanism. There is still current debate and investigation of the mechanistic details of the involvement of the thiamine ylid 1 in biological systems, including the role of the associated metal ions (15, 30).

Thiamine is susceptible to reaction with various nucleophilic species. During the race for the isolation of thiamine, it was found by chance during attempted stabilization of extracts that thiamine is readily ruptured by sulfite treatment. This occurs slowly at pH 3 and rapidly at pH 5 and above, to give 4-amino-2-methyl-5-pyrimidine-methanesulfonic acid [108084-76-0] 4 (Fig. 4), with liberation of the thiazole moiety **(8)** (2, 10).

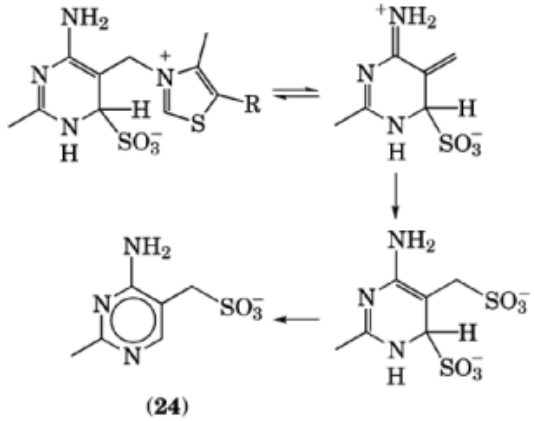


Fig. 4. Mechanism of nucleophilic degradation.

Other nucleophilic groups such as pyridines, phenolates, anilines, and azide react similarly in the required presence of sulfite. An addition–elimination mechanism involving sulfite attack on the pyrimidine ring has been elucidated (9, 10). Similar reactions are observed in the degradation of thiamine by thiaminases, enzymes found in certain foods and bacteria, including some found in the human intestine (1, 10, 31). Thiaminase I (EC 2.5.1.2), found in shellfish, ferns, some vegetables and some bacteria, promotes the replacement of the thiazole by other organic bases, including purines. Thiaminase II (EC 3.5.99.2), found mostly in bacteria, catalyzes the cleavage of thiamine into **(7)**, the biosynthetic precursor of thiamine and an antagonist of pyridoxine (vitamin B_6). A thiol group at the active site is strongly implicated (11). These enzymes can have strong effects on animals as they promote thiamine deficiency (32). Fortunately, they are thermolabile, and hence a problem mostly in uncooked foods or feeds.

Thermostable substances which inactivate thiamine have been found in a large number of plants and in some animal tissues. Among these are polyphenols such as caffeic acid, tannic acid, hydroxylated derivatives of tyrosine, and some flavonoids (1). Thiamine is susceptible to destruction by x-rays, gamma rays, and ultraviolet light to generate diamine 2 by cleavage of the thiazole ring (1). Photochemical and thermal degradation of thiamine gives rise to numerous sulfur-containing heterocycles, some with meaty or bread aromas of interest to the flavor industry (33).

Operations in preparing or preserving food and feeds can sometimes lead to significant losses of thiamine value through leaching, chemical destruction (from high heat, high energy irradiation, or especially alkaline oxidizing conditions), and specific chemical interactions with thiamine-destroying substances. Such losses range in amounts from a few percent to as high as 85%. In many cases, the destruction of thiamine shows apparent first-order kinetics, and Arrhenius calculations can be used to estimate losses (1, 34).

Thiamine forms the expected derivatives of the thiazole alcohol function, such as carboxylic and phosphate esters. Few reactions at the pyrimidine 4-amino function have been reported. Most of the usual conditions used for formation of amides, for example, lead to destruction of the thiazolium ring.

2. Natural Occurrence

Thiamine is widespread in nature, although generally in only relatively minute quantities (1, 35). In microorganisms it is found mainly intracellularly, although minute amounts are lost to the natural environment upon cell lysis. In higher plants the most abundant form is free thiamine along with lesser amounts of the phosphate

Table 1. Average Thiamine Contents of Foods

Food	Thiamine, μg/100 g
wheat germ	2050
dried brewer's yeast	1820
soybeans	1300
pork	600–950
dried beans and peas	680
dried milk whey	500
nuts	300–560
brown rice	300
beef liver	300
potatoes	170
fish	50–90
eggs	70
vegetables (fruit, leaf, stem, root)	60–70
whole milk	30–70
white rice	50

esters **(4–6)**. Within the plant, thiamine is unevenly distributed, its amounts and location depending on the life stage. Seeds and uptake from the soil supply the plant until biosynthesis in the leaves begins. Thiamine is then transported from the leaves to the roots where it exhibits hormone-like effects on their growth. Later thiamine is again concentrated in the seeds, especially in the germ and in the pericarpal layers surrounding the starchy areas of the seed (36). As the germ and bran is often removed during processing for aesthetic reasons, highly refined rice or wheat, for example, can have significantly lowered contents of thiamine, and can be a cause of thiamine deficiency when used as staple foods.

In the tissues of animals, most thiamine is found as its phosphorylated esters **(4–6)** and is predominantly bound to enzymes as the pyrophosphate **(5)**, the active coenzyme form. As expected for a factor involved in carbohydrate metabolism, the highest concentrations are generally found in organs with high activity, such as the heart, kidney, liver, and brain. In humans this typically amounts to 1–8 μg/g of wet tissue, with lesser amounts in the skeletal muscles (35). A typical healthy human body may contain about 30 mg of thiamine in all forms, about 40–50% of this being in the muscles owing to their bulk. Almost no excess is stored. Normal human blood contains about 90 ng/mL, mostly in the red cells and leukocytes. A value below 40 ng/mL is considered indicative of a possible deficiency. Amounts and proportions in the tissues of other animal species vary widely (31, 35).

Good natural human dietary sources of thiamine are unrefined cereal grains, organ meats, pork, legumes, and nuts (Table 1). Oils, fats, and highly refined foods are essentially devoid of thiamine (3, 36). Although thiamine is widespread in foods, some can be lost in food preparation and storage. As a result, dietary intake can vary significantly. In most developed countries, foods (typically white rice and white flour) and feeds are supplemented with thiamine and its use in vitamin tablets as dietary supplements is common. Enriched grains, cereals, and baked products contribute about 30–45% of the recommended daily allowance (RDA) for the adult diet in the United States.

3. Biochemical and Physiological Functions

Thiamine serves essential functions and its deficiency causes particularly deleterious effects on an organism's energy status and, in higher organisms, its nerve functions. In living systems, the only established biochemically

Table 2. U.S. RDAs for Thiamine

Population segment	RDA, mg
infants and children <4 yr	0.7
adults and children >4 yr	1.5
pregnant or lactating women	1.7

active form is the pyrophosphate (cocarboxylase) **(5)**, which plays a vital role in intermediate metabolism as a cofactor for some important enzymatic reactions. Dehydrogenase enzymes require cocarboxylase **(5)** for oxidative decarboxylation of 2-ketoacids, notably pyruvate in glycolysis, 2-oxoglutarate in the citric acid cycle, and other ketoacids from amino acid decarboxylation (1). Transketolase enzymes require cocarboxylase **(5)** for the reversible transfer of alpha-ketols in ketose–aldose transformations important in the production of pentoses for RNA and DNA synthesis (1). Thiamine triphosphate **(6)** occurs in unusually higher concentrations in nerve tissues and the brain and may play an essential role in the stimulation of peripheral nerves (35).

In humans, thiamine is both actively and passively absorbed to a limited level in the intestines, is transported as the free vitamin, is then taken up in actively metabolizing tissues, and is converted to the phosphate esters via ubiquitous thiamine kinases. During thiamine deficiency all tissues stores are readily mobilized. Because depletion of thiamine levels in erythrocytes parallels that of other tissues, erythrocyte thiamine levels are used to quantitate severity of the deficiency. As deficiency progresses, thiamine becomes indetectable in the urine, the primary excretory route for this vitamin and its metabolites. Six major metabolites, of more than 20 total, have been characterized from human urine, including thiamine fragments **(7,8)**, and the corresponding carboxylic acids (1, 37, 38).

The classic pathology resulting from severe thiamine deficiency in humans is called beriberi. Similar conditions have been described for many other animals. Beriberi develops primarily from inadequate intake of thiamine or from ingestion of food containing antithiamine factors and is somewhat rare in developed areas of the world. Less severe thiamine deficiency is more common and is characterized by anorexia and mental disturbances, such as irritability, inattention, memory defects, depression, and insomnia. If a lesser deficiency is left untreated, one of several clinical forms of beriberi develops, the symptoms of which include mental changes, peripheral neuritis, paresthesias, muscle cramps, edema, muscular atrophy, and cardiac failure. The most commonly encountered type of thiamine deficiency in Western countries is associated with alcohol abuse. This is generally thought to result from high intake of empty calories and low intake of nutritionally adequate foods. Other factors that influence thiamine status include general level of muscular activity; dietary practices such as high intake of refined carbohydrates, tea or coffee, or raw seafood; reduced thiamine intake as a result of disease, or parasites or drugs lowering food intake and utilization or thiamine absorption; pregnancy and lactation; heavy smoking; advanced age; genetic background; and stress. In other animals, climate and intestinal microflora also become important, and toxic effects can occur from changes in the microflora (39). Thiamine status has been monitored by blood thiamine levels, thiamine and metabolite levels in the urine, blood pyruvate and lactate levels, and blood transketolase activity (1, 37, 38).

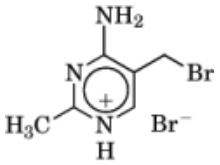
Thiamine requirements vary and, with a lack of significant storage capability, a constant intake is needed or deficiency can occur relatively quickly. Human recommended daily allowances (RDAs) in the United States are based on calorie intake at the level of 0.50 mg/4184 kJ (1000 kcal) for healthy individuals (Table 2). As little as 0.15–0.20 mg/4184 kJ will prevent deficiency signs but 0.35–0.40 mg/4184 kJ are required to maintain near normal urinary excretion levels and associated enzyme activities. Pregnant and lactating women require higher levels of supplementation. Other countries have set different recommended levels (1, 37, 38).

4. Manufacture

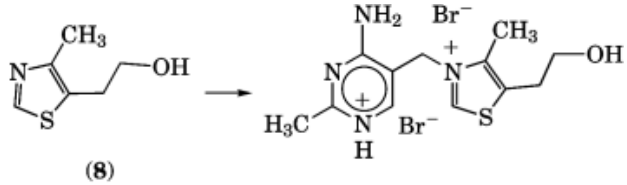
4.1. Chemistry

Isolation of thiamine from natural sources (rice bran, yeast extracts, or wheat germ) is only of historical interest. Production by bioprocesses is not cost-effective at present. All of the thiamine produced worldwide is manufactured by chemical processes operated at moderately large scale. Two major synthetic routes have been used: alkylation of a preformed thiazole, or construction of the thiazolium salt from a pyrimidine carrying the ultimate thiazole nitrogen (9, 40). The latter approach is now generally preferred for manufacturing.

The first approach parallels the known biosynthetic pathway where the alkylating agent is the pyrophosphate ester of alcohol **(7)**. Typical of this approach is Williams' first convergent synthesis, which is the basis for the first industrial method developed by Merck & Co. (2, 5, 41). Synthetic 4-amino-5bromomethyl-2-methylpyrimidine [2908-71-6] **(25)** and thiazole **(8)**, or its *O*-acetate, were condensed and then the bromide was replaced by use of silver salts. Numerous variants were published or patented in the 1930s and 1940s. Such methods generally gave only moderate yields, used higher temperatures in polar solvents, required tedious purification of the colored products and are no longer used.



(**26**)



(**8**)

In the second general method, the amine 2, known as Grewe diamine, is the paradigm intermediate onto which the thiazolium ring is constructed. Differences occur only in the raw materials and methods used for the manufacture of the diamine. The same end of the linear sequence is universally practiced by all large manufacturers.

The synthesis which forms the basis of production at Hoffmann-La Roche (Fig. 5) proceeds via the pyrimidinenitrile [698-29-3] 5 made from malononitrile, trimethylorthoformate, ammonia, and acetonitrile (42, 43). High pressure catalytic reduction of the nitrile furnishes diamine 2. The overall sequence is short, highly efficient, and generates almost no waste. However, malononitrile is a relatively expensive and hazardous three-carbon source.

Other syntheses produce Grewe diamine using inexpensive acrylonitrile and alkyl formates as raw materials. Such routes deliver the pyrimidine bridge carbon at the correct aminomethyl oxidation level without a need for reduction. Thus, in the older method of Shionogi, base-catalyzed acylation of 3-methoxypropanenitrile followed by enolate alkylation and ring closure with two equivalents of acetamidine gives the acetyl derivative [23676-63-3] 6 of Grewe diamine (Fig. 6) (44–46). Alternatively, in a newer method by BASF, acylation of 3-formamidopropanenitrile, followed by ring closure with acetamidine, gives diamine as its formamide [1886-34-6] 7 (Fig. 7) (47–50). Both the Shionogi and BASF methods are simple technologies based on inexpensive raw materials, but both generate significant levels of salts and organic carbon as wastes.

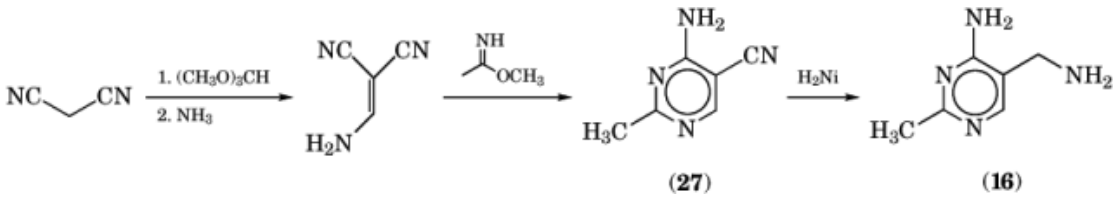


Fig. 5. Roche process.

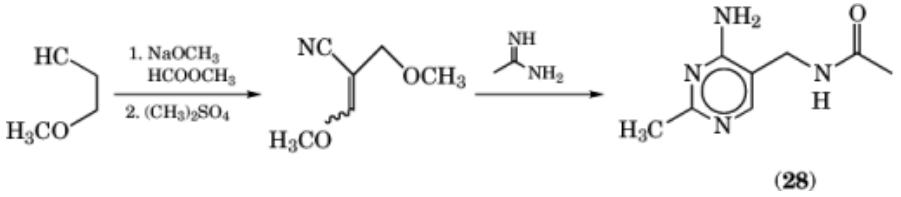


Fig. 6. Shionogi process.

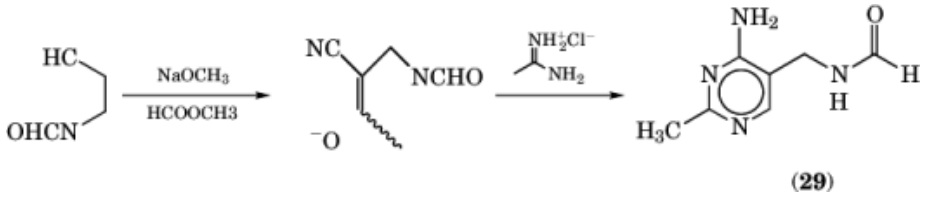


Fig. 7. BASF process.

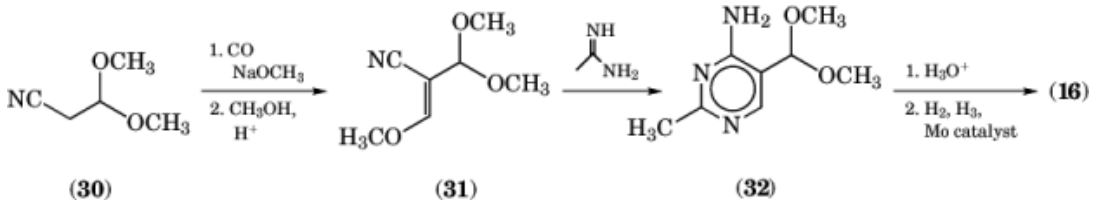
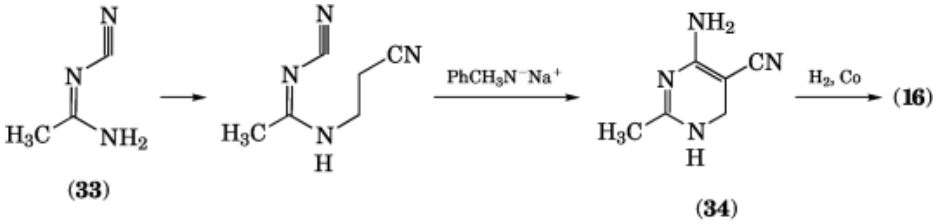


Fig. 8. Takeda-Ube process.

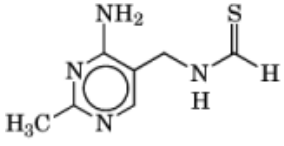
In the past decade Takeda and Ube, in a joint venture have developed a new process for diamine, also based on acrylonitrile and alkyl formate, but carrying the pyrimidine bridge carbon at the carbonyl level (Fig. 8) (50–55). Metal-catalyzed oxidation of acrylonitrile in methanol generates 3,3-dimethoxypropanenitrile [57597-62-3] 8 which is acylated with methoxide/carbon monoxide under pressure. Unlike other methods in which the intermediate enolate is alkylated, in this process the enolate is acetalized and the acetal thermally converted to the acetal enol ether [87466-78-2] 8. Condensation with acetamidine provides the remaining carbons. After hydrolysis of the acetal [16057-06-0] 8, reductive amination at high pressure with a specialized catalyst provides diamine 2 in very high overall yield. The process is operated in a new, large-scale, automated, continuous, technically complex plant in Japan. Advantages include use of inexpensive acrylonitrile and carbon monoxide, avoidance of the costs of an alkylating agent, and consumption of only one equivalent of acetamidine.

In a much different approach based on cyanamide, acrylonitrile, and acetonitrile, cyanoacetamidine [56563-07-6] **(32)** is cyanoethylated and the condensation product [56563-10-1] **(33)** is dehydrogenated and hydrogenated directly to Grewe diamine in the presence of Raney cobalt and ammonia (56).

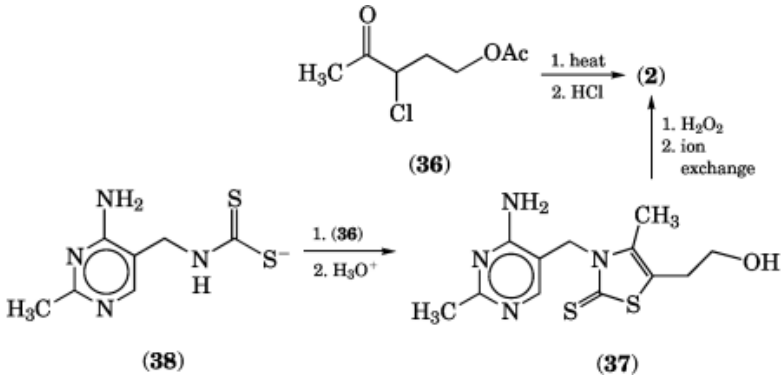


(33) (34)

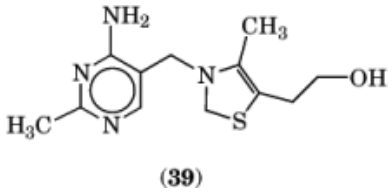
Methods for synthesis of the thiazolium ring also have matured technically based on the cost, throughput, and waste disposal issues of production. In earlier syntheses, the 2-carbon and the sulfur atom were supplied as potassium dithioformate, made from chloroform and potassium sulfide. *N*-(4-Amino-2-methyl-5-pyrimidinyl)-methylthioformamide [31375-20-9] **(34)** served as a partner to 5-acetoxy-3-chloropentan-2-one [119867-66-2] **(35)** in a classical Hantzch thiazole synthesis (3, 57). Such routes suffered from similar limitations to the quaternization routes mentioned before, but were used in commercial production until the discovery of the paradigm intermediate known as thiothiamine [299-35-4] **(36)**. In the 1940s and 1950s, chemists at Tanabe and Takeda showed that the 2-carbon and the sulfur atom can be supplied very efficiently via carbon disulfide, the extra sulfur atom being readily removed by oxidation in nearly quantitative yield (58, 59). Typically, an aqueous solution of diamine and alkali is treated in succession with carbon disulfide to form the dithiocarbamate [2882-49-7] **(37)**, then with chloroketone **(35)**, then acid to form the relatively insoluble, thiothiamine **(36)**. Oxidation with hydrogen peroxide forms thiamine sulfate, which is converted by ion exchange (qv) to a solution of the hydrochloride **(2)** which is concentrated, crystallized, and dried. The much less soluble nitrate **(3)** is precipitated from aqueous solution with alkali metal nitrate. The advantages of this approach, ie, a cheap, easily handled sulfur source, very high overall yield and throughput, excellent product color and purity, and the use of water as solvent, have made it the preferred method of manufacture worldwide. Disadvantages include use of chlorinated intermediates, salt load from the sulfate waste stream, and malodorous aqueous waste which must be well contained and treated. This chemistry is practiced by Takeda and Hoffmann-La Roche in automated factories using standard glass and stainless steel fine chemical processing equipment operated continuously at many hundreds of metric tons annually. Production in the several smaller Chinese factories is probably basically similar.



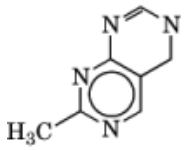
(35)



In two unique, convergent approaches to the thiazole ring, other formate synthons are used for the 2-carbon. In one, reaction of formaldehyde with diamine 2 and thiol 2 gives dihydrothiamine [959-18-2] **(38)**, which is oxidized to thiamine (60). In another, reaction of diamine 2 with orthoformate gives intermediate dihydropyrimidine [31375-19-6] **(39)**, to which thiol 2 is added. Acidic rearrangement gives thiamine in high yield (60).

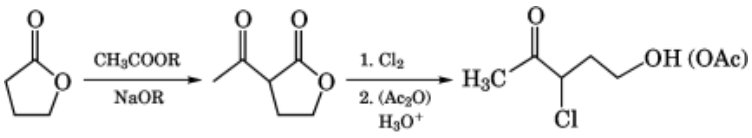


(39)



(40)

Although numerous other materials have been proposed as carbon sources for the thiazole ring, all manufacturing of thiamine is believed to use 3-chloro-5-acetoxypentan-2-one **(35)** or the corresponding alcohol [13045-13-1] **(40)** as intermediates. These are made by chlorination of acetylbutyrolactone, the latter from inexpensive butyrolactone and methyl acetate, generating chlorinated wastes.



(**36**, OAc)
(**41**, OH)

Worldwide production of thiamine was estimated at 3300 t in 1995. The principal suppliers were Hoffmann-La Roche, Takeda, and several Chinese factories. Prices in the United States were in the range of $20–$28/kg in 1995.

4.2. Product Specifications and Testing

Nutrients and diet supplements without claims are considered foods, and thus are regulated by the U.S. Food and Drug Administration and are further subject to specific food regulations. Specifications for the hydrochloride **(2)** and the mononitrate **(3)** for foods are given in the *Food Chemicals Codex* (62) and for pharmaceuticals in the *U.S. Pharmacopeia* (63). General test methods have been summarized (64).

4.3. Safety and Handling

The hydrochloride **(2)** and the nitrate **(3)** are typically packaged in standard 20–25-kg foil or poly-lined cardboard boxes. Smaller packaging sizes are also available. Both hydrochloride **(2)** and nitrate **(3)** are required to be kept under normal cool, dry storage conditions, not to exceed 70°C and protected from light. Both are listed in the TSCA Inventory and material safety data sheets (MSDS) are available from suppliers. Precautions against dust exposure to the eyes, skin, or lungs (IOEL of 3.0 mg/m^3 time weighted average) and against fire and dust explosion are indicated. The NPFA ratings for health, fire, and reactivity for the hydrochloride **(2)** are 1, 2, and 1, respectively, and for the nitrate **(3)** they are 1, 3, and 1. Neither national nor international transportation is regulated. The U.S. EPA has not established reportable quantities for environmental releases (7, 8). The acute LD_{50} (rat, oral) of 12,300 mg/kg for the hydrochloride **(2)** and 15,900 for the nitrate **(3)** qualify these materials as relatively harmless orally. There is no evidence of toxicity from thiamine taken orally by humans, even when doses of 500 mg, over 300 times the RDA, are taken daily for a month. Excess thiamine is easily cleared by the kidney (7, 8, 65). Parenteral doses of the hydrochloride (subcutaneous, intramuscular, or intravenous) have generally been well tolerated up to 100–500 mg with very few toxic effects. Some cases of sensitization or anaphylactic shock on repeated injections have been reported (1).

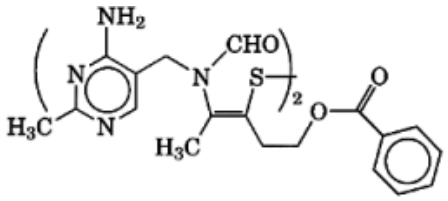
5. Analytical Methods

Fluorometric, chromatographic, microbiological, and animal assays have been used for thiamine and its derivatives (66). The most widely used and officially sanctioned method has been the fluorometric assay (67), although high performance liquid chromatography has been increasingly employed (68). In natural materials, thiamine is often present as its phosphate esters and is protein-bound, therefore procedures to free it are necessary steps in most assays. Determination of thiamine by the fluorometric method involves acid extraction, phosphatase hydrolysis, ion exchange to remove interferences, and oxidation with ferricyanide or other reagents to thiochrome, whose fluorescence (excitation 365 nm/emission 435 nm) is measured and compared with a standard (67). Various hplc methods have been developed for quantitation of thiamine and its phosphates in biological matrices to picomole to femtomole levels. Separations are made with reversed-phase, ion-exchange, and specialized straight-phase packings and detection with uv, post-column derivatization–fluorometry, or electrochemical techniques. Alternatively, precolumn derivatization and measurement of thiochrome has been used (68). High performance capillary electrophoresis has been applied (69). Gas chromatography has been used to determine thiamine to picomole level as the thiazole portion following cleavage of thiamine with sulfite (71). Microbiological assays are simple, inexpensive, and sensitive (5–50 ng thiamine). The results agree well with fluorometric methods but can take longer to achieve results. Microorganisms that have been most widely used include *Lactobaccillus fermenti* (ATCC 9338) and *Lactobacillus viridescens* (ATCC 12706), partly because they respond only to intact thiamine and not to its precursors (31). Bioassays in rats based on growth or cure of

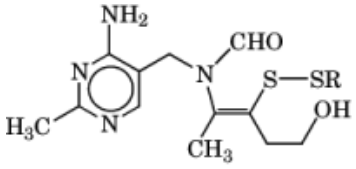
polyneurititis symptoms are time consuming and expensive but have been historically valuable in determining thiamine availability in foods and thiamine activity of related compounds (31).

6. Forms, Derivatives, and Analogues

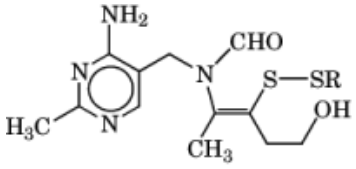
The hydrochloride and mononitrate are the only commercial forms approved in the United States. The mono-, di-, and triphosphate esters **(4–6)** are colorless, water-soluble, organic-insoluble, high melting solids found naturally. Although they have been synthesized (9), they have not been produced commercially on large scale. In Japan and Europe, other forms have been approved for human use. Thiamine disulfide 2 is somewhat more soluble than thiamine in organic media. This material and its more fat-soluble *O,O*-dibutyrate and *O,O*-dibenzoate [2667-89-2] **(41)** have been used therapeutically for treatment of thiamine deficiency (70). Treatment of thiamine with extracts of garlic or other *Allium* species converts it to a lipid-soluble disulfide derivative which is a very physiologically active depot form of thiamine. Thiamine allyl disulfide [554-44-9] (allithiamine) **(42)**, was the first of several thiamine alkyl disulfides to be used therapeutically for thiamine deficiency, including prosultiamine [59-58-5] **(43)** and fursultiamine [804-30-8] **(44)**. Because of their greater lipid solubility, they are absorbed and retained more strongly in the body. *In vivo* they are converted back to thiamine. They are of interest almost exclusively in Japan and are not yet approved in the United States (70, 71). Sugar derivatives (qv) are claimed to have better taste and no odor (73). In applications where water solubility is detrimental, such as fish feeds with high thiaminase activity, formulations of the hydrochloride or nitrate with a waxy coating are effective.



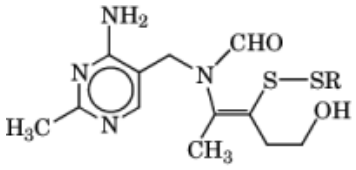
(42)



(43), allyl

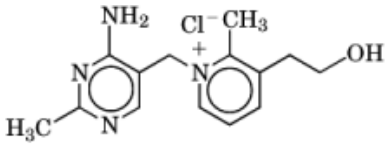


(44), propyl

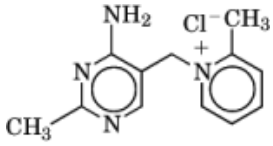


(**45**), tetrahydrofurfuryl

Numerous analogues of thiamine have been synthesized by structural modifications of the pyrimidine or thiazole rings or the bridging atoms (1, 9, 10). None has been found to exceed the biological activity of thiamine but some act as antagonists. Substitution at the 2-methyl, 4-amino, or 6-position of carbon for ring nitrogens, or of the methylene bridge, results in loss of activity. In the thiazolium ring, the sulfur atom, hydrogen at the 2-position, and the 2-hydroxyethyl group are essential for activity. The pyridine analogue, pyrithiamine [534-64-5] **(45)** is the most potent thiamine antagonist known. Some thiamine antagonists were found at low levels to selectively inhibit thiamine uptake by *Coccidia*. A modified thiamine-like molecule, called Amprolium [121-25-5] **(46)** was once claimed to be useful as a coccidiostat for poultry (73).



(**46**)



(**47**)

7. Biosynthesis

Higher plants, most bacteria, and some fungi biosynthesize thiamine. Humans or most other animals cannot, although some of their gut microflora can. Many microbial species are self-sufficient, others can synthesize thiamine if one or both of immediate precursors are available, and still others require the complete substance. Few produce much of an excess over their own requirements. Biosynthesis of thiamine in microorganisms has been reviewed (9, 74–76). Media modifications and mutagenesis have achieved small increases of thiamine levels with microorganisms, the highest levels of a few mg/L being found with yeasts (qv) (77). A broader search among many yeast species has reported a *Saccharomyces cerevisiae* strain which accumulates up to 200 mg/L in the culture broth (78).

The pathways for thiamine biosynthesis have been elucidated only partly. Thiamine pyrophosphate is made universally from the precursors 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate [841-01-0] 9 and 4-methyl-5-(2-hydroxyethyl)thiazole phosphate [3269-79-2] 9, but there appear to be different pathways in the earlier steps. In bacteria, the early steps of the pyrimidine biosynthesis are same as those of purine nucleotide biosynthesis, 5-Aminoimidazole ribotide [41535-66-4] (AIR) 9 appears to be the sole and last common

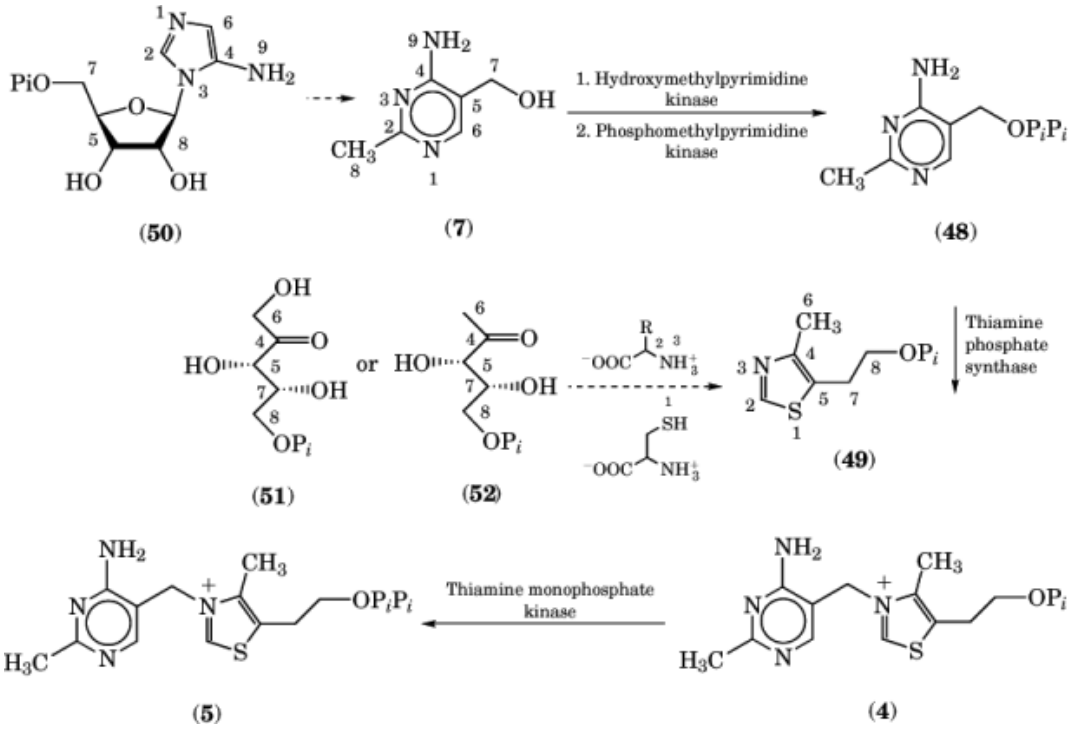


Fig. 9. Biosynthesis of thiamine.

intermediate; ultimately the elements are supplied by glycine, formate, and ribose. AIR is rearranged in a complex manner to the pyrimidine by an as-yet undetermined mechanism. In yeasts, the pathway to the pyrimidine is less well understood and may be different (74–83) (Fig. 9).

In the biosynthesis of the thiazole, cysteine is the common sulfur donor. In yeasts, the C-2 and N may be supplied by glycine, and the remaining carbons by D-ribulose-5-phosphate [108321-99-9] 9. In anaerobic bacteria, the C-2 and N may be recruited from tyrosine and the carbons from D-1-deoxyxylulose [16709-34-5] 9, whereas in aerobic bacteria the C-2 and N may be derived from glycine, as in yeasts 7 (74–76, 83–86) (see Fig. 9).

Biosynthesis of pyrophosphate **(5)** from pyrimidine phosphate 9 and thiazole phosphate 9 depends on the activity of five enzymes, four of them kinases (87). In yeasts and many other organisms, including humans, pyrophosphate **(5)** can be obtained from exogenous thiamine in a single step catalyzed by thiamine pyrophosphokinase (88).

A number of the genes involved in the biosynthesis of thiamine in *E. coli* (89–92), *Rhizobium meliloti* (93), *B. subtilis* (94), and *Schizzosaccharomyces pombe* (95, 96) have been mapped, cloned, sequenced, and associated with biosynthetic functions. Thiamine biosynthesis is tightly controlled by feedback and repression mechanisms limiting overproduction (97, 98). A cost-effective bioprocess for production of thiamine will require significant additional progress.

8. Uses

Most of the thiamine sold worldwide is used for dietary supplements. Primary market areas include the following applications: addition to feed formulations, eg, poultry, pigs, cattle, and fish (see Feeds and feed additives); fortification of refined foods, eg, flours, rice, and cereal products; and incorporation into multivitamins. Small amounts are used in medicine to treat deficiency diseases and other conditions, in agriculture as an additive to fertilizers (qv), and in foods as flavorings. Generally for dry formulations, the less soluble, nonhygroscopic nitrate is preferred. Only the hydrochloride can be used for intravenous purposes. Coated thiamine is used where flavor is a factor.

BIBLIOGRAPHY

"Thiamine" in *ECT* 1st ed., Vol. 14, pp. 38–48, by E. Pierson, Merck & Co., Inc; "Thiamine" under "Vitamins" in *ECT* 2nd ed., Vol. 20, pp. 173–184, by P. I. Pollak, Merck & Co., Inc.; in *ECT* 3rd ed., Vol. 24, pp. 124–139, by Y. Oka, Takeda Chemical Industries, Ltd.

Cited Publications

1. C. J. Gubler, in L. J. Machlin, ed., *Handbook of Vitamins*, 2nd ed., Marcel Dekker, New York, 1991, 233–280.
2. R. R. Williams and J. K. Cline, *J. Am. Chem. Soc.* **58**, 1504 (1936); *ibid.*, **59**, 1052 (1937).
3. A. R. Todd and F. Bergel, *J. Chem. Soc.*, 364 (1936).
4. A. R. Todd and F. Bergel, *J. Chem. Soc.*, 26 (1938).
5. H. Andersag and K. Westphal, *Ber. Dtsch. Chem. Ges.* **70**, 2035 (1937).
6. *The Merck Index*, 11th ed., Merck & Co., Rahway, N.J., 1989, p. 1464.
7. *Thiamine Hydrochloride*, MSDS 3834, Hoffmann-La Roche Inc., Nutley, N.J.
8. *Thiamine Mononitrate*, MSDS 3835, Hoffmann-La Roche Inc., Nutley, N.J.
9. O. Isler, G. Brubacher, S. Ghisla, and B. Kraeutler, *Vitamine II*, Georg Thieme Verlag, New York, 1988, 17–49.
10. J. A. Zoltewicz and G. Uray, *Bioorganic Chem.* **22**, 1–28 (1994) and references therein.
11. J. M. E. H. Chahine and J. E. Dubois, *J. Am. Chem. Soc.* **105**, 2335 (1983).
12. J. D. Roberts and co-workers, *J. Am. Chem. Soc.* **99**, 6423 (1977).
13. A. Lycka and J. Cizmarik, *Pharmazie* **45**, 371 (1990).
14. J. M. E. H. Chahine, *J. Chem. Soc., Perkin Trans. II*, 505 (1990).
15. R. Kluger, *Chem. Rev.* **87**, 863 (1987).
16. R. D. Sedgwick and co-workers, *J. Chem. Soc., Chem. Commun.*, 325 (1987).
17. J. J. Windheuser and T. Higuchi, *J. Pharm. Sci.* **51**, 3545 (1962).
18. T. Matsukawa and S. Yurugi, *Yakugaku Zasshi* **71**, 827 (1951).
19. J. Herrmann and co-workers, *J. Chem. Soc., Perkin Trans. II*, 463 (1995).
20. A. Watanabe and Y. Asahi, *Yakugaku Zasshi* **77**, 153 (1957).
21. A. Takamizawa and co-workers, *Chem. Pharm. Bull.* **19**, 759 (1971).
22. T. Matsukawa and S. Yurugi, *Yakugaku Zasshi*, **72**, 1599 (1952).
23. H. Sugimoto, K. Hirai, and co-workers, *J. Org. Chem.* **55**, 46 (1990).
24. J. Fournier, *Bull. Soc. Chim. Fr.*, 854 (1988).
25. J. M. E. H. Chahine and J. E. Dubois, *J. Chem. Soc., Perkin Trans. II*, 25 (1989).
26. Y.-T. Chen and F. Jordan, *J. Org. Chem.* **56**, 5029 (1988).
27. R. Breslow, *J. Am. Chem. Soc.* **80**, 3719 (1958).
28. J. T. Stiners and M. W. Washabaugh, *Bioorg. Chem.* **18**, 425 (1990).
29. R. Breslow, *Pure Appl. Chem.* **62**, 1859–1866 (1990).
30. M. Louloudi and N. Hadjiliadis, *Coord. Chem. Rev.* **135/136**, 429–468 (1994).
31. W. H. Sebrell and R. S. Harris, eds., *The Vitamins*, 2nd ed., Vol. **V**, Academic Press, Inc., New York, 1972, p. 98.

32. W. C. Evans, in P. L. Munson, J. Glover, E. Diezfalusy, and R. E. Olson, eds., *Vitamins and Hormones*, Vol. **33**, Academic Press, Inc., New York, 1975, 467–504.
33. P. Werkhoff and co-workers, *ACS Symp. Ser.* **564**, 199–223 (1994).
34. L. Mauri and co-workers, *Int. J. Food Sci. Technol.* **24**, 1–9 (1989).
35. M. P. Lamden, in W. H. Sebrell, Jr. and R. S. Harris, eds., *The Vitamins*, 2nd ed., Vol. **V**, Academic Press, Inc., New York, 1972, 114–120.
36. A. Mozafar, *Plant Soil* **167**, 305–311 (1994).
37. R. E. Davis and G. C. Icke, *Adv. Clin. Chem.* **23**, 93 (1983).
38. P. M. Finglas, *Int. J. Vit. Nutr. Res.* **63**, 270 (1993).
39. J. Harmeyer and U. Kollenkirchen, *Nutr. Res. Rev.* **2**, 201–225 (1989).
40. D. Brown and co-workers, *The Pyrimidines*, 2nd ed., John Wiley & Sons, Inc., New York, 1994.
41. U.S. Pat. 2,235,862 (Mar. 25, 1940), O. Zima (to Merck & Co.).
42. R. Grewe, *Z. Physiol. Chem.* **242**, 89 (1936).
43. Fr. Pat. 831,110 (Aug. 23, 1938), (to Hoffmann-La Roche).
44. A. Takimizawa and K. Hirai, *Chem. Pharm. Bull.* **12**, 393 (1964).
45. H. Miromoto and co-workers, *Chem. Ber.* **106**, 893 (1973).
46. K. Tokuyama and co-workers, *Bull. Chem. Soc. Jap.* **46**, 253 (1973).
47. U.S. Pat. 4,226,799 (Oct. 16, 1978), W. Bewert and W. Littmann (to BASF).
48. Ger. Pat. 3,511,273 (Mar. 28, 1985), W. Ernst and J. Paust (to BASF).
49. Ger. Pat. 3,520,982 (Dec. 18, 1986), H. Kiefer and W. Bewert (to BASF).
50. K. Nishihara and co-workers, *Kagaku Kogaku* **55**, 433 (1991).
51. Eur. Pat. 55,108 (June 30, 1982), K. Matsui and co-workers (to Ube Industries).
52. Eur. Pat. 279,556 (Aug. 24, 1988), K. Nishihara and co-workers (to Ube Industries).
53. U.S. Pat. 4,536,577 (Aug. 20, 1985), H. Yoshida and S. Niida (to Ube Industries).
54. Ger. Pat. 3,303,789 (Aug. 11, 1983), H. Yoshida and co-workers (to Ube Industries).
55. Rus. Pat. 6,307,869 (July 10, 1988), Y. Miyashiro and co-workers (to Takeda Chemical Industries).
56. A. Edenhoffer and co-workers, *Helv. Chim. Acta* **58**, 1230 (1975).
57. T. Matsukawa and co-workers, *J. Pharm. Soc. Japan* **63**, 216 (1943).
58. K. Washimi, *J. Pharm. Soc. Japan.* **66**, 62 (1946).
59. T. Matsukawa and S. Hojiro, *J. Pharm. Soc. Japan* **69**, 550 (1949); *ibid.*, **70**, 28 (1950); **71**, 667, 720, 1215 (1951).
60. H. Hirano and Y. Yonemitsu, *J. Pharm. Soc. Japan* **76**, 1332 (1956).
61. G. Moine and co-workers, *Helv. Chim. Acta* **73**, 1300 (1990).
62. Food and Nutrition Board, National Research Council, *Food Chemicals Codex*, 3rd ed., National Academy Press, Washington, D.C., 1981, 324–325.
63. The United States Pharmacopeia XXII (USP XXII-NF XVII) United States Pharmacopeial Convention, Inc., Rockville, Md., 1990, pp. 1356, 1357–1358.
64. F. J. Al-Shammary and co-workers, *Anal. Profiles Drug Subst.* **18**, 414 (1989).
65. H. E. Samerlich and L. J. Machlin, *Ann. NY Acad. Sci.* **699**, 1 (1992).
66. W. C. Ellefson, in J. Augustine, B. P. Klein, D. A. Becker, and P. B. Venugopal, eds., *Methods of Vitamin Assay*, 4th ed., John Wiley & Sons, Inc., New York, 1985, 351–352.
67. K. Helrich, ed., *Official Methods of Analysis of the Association of Official Analytical Chemists*, 15th ed., Association of Official Analytical Chemists, Inc., Arlington, Va., 1990, pp. 942.23, 953.17, 957.17, and 986.27.
68. T. Kawasaki, in A. P. Leenheer, W. E. Lambert, and M. G. M. DeRuyter, eds., *Modern Chromatographic Analysis of the Vitamins*, Marcel Dekker, New York, 1992, 319–354.
69. U. Jegle, *J. Chrom.* **652**, 495–501 (1993).
70. C. Kawasaki, in R. Hamb, I. Wool, and J. Lomaine, eds., *Vitamins and Hormones*, Vol. **21**, Academic Press, Orlando, Fla., 1963, 69–111.
71. R. E. Echols and co-workers, *J. Chrom.* **347**, 89–97 (1985).
72. C. E. Mueller and co-workers, *Int. J. Pharm.* **57**, 41–47 (1989).
73. Y. Susuki and K. Uchida, *Biosci. Biotech. Biochem.* **58**, 1273 (1994).
74. W. H. Ott and co-workers, *Poult. Sci.* **44**, 920 (1965).
75. A. Iwashima in E. Vandamme, ed., *Biotechnology of Vitamins, Pigments and Growth Factors*, Elsevier, Amsterdam, the

Netherlands, 1988.
76. D. W. Young, *Nat. Prod. Rep.* **3**, 395 (1986).
77. G. M. Brown and J. M. Williamson, in F. C. Neidhardt, ed., *Escherichia coli and salmonella typhimurium*, American Society for Microbiology, Washington, D.C., 1987, p. 521.
78. A. Silhamkeva, *J. Inst. Brew.* **191**, 78 (1985).
79. G. G. Stewart and co-workers, *Can. J. Microbiol.* **38**, 1156 (1992).
80. T. Kozlok and I. D. Spenser, *J. Am. Chem. Soc.* **109**, 4968 (1987).
81. D. M. Downs and L. Peterson, *J. Bacteriol.* **176**, 4858 (1994).
82. N. J. Leonard and co-workers, *Proc. Natl. Acad. Sci.* **85**, 7174 (1988).
83. B. Estramareix and co-workers, *Biochem. Biophys. Acta* **1035**, 154 (1990).
84. K. Tazuya and co-workers, *Biochem. Mol. Biol. Int.* **30**, 893 (1993).
85. K. Tazuya and co-workers, *Biochem. Int.* **10**, 689 (1985).
86. R. H. White and F. B. Rudolph, *Biochem. Biophys. Acta* **542**, 340 (1978).
87. K. Tazuya and co-workers, *Biochem. Int.* **14**, 153 (1987).
88. Y. Kawasaki, *J. Bacteriol.* **175**, 5153 (1993).
89. A. Iwashima and co-workers, *J. Biol. Chem.* **268**, 17440 (1993).
90. T. Nohno, Y. Kasai, and T. Saito, *J. Bacteriol.* **170**, 4097 (1988).
91. N. Imamura and H. Nakayama, *J. Bacteriol.* **151**, 708 (1982).
92. J. Ryals and co-workers, *J. Bacteriol.* **151**, 899 (1982).
93. T. P. Begley and co-workers, *J. Am. Chem. Soc.* **117**, 2351 (1995).
94. H. Brennen and co-workers, *J. Bacteriol.* **167**, 66 (1986).
95. J. A. Hoch and co-workers, in A. L. Sonenshein, J. A. Hoch, and R. Losick, eds., *Bacillus subtilis*, American Society for Microbiology, Washington, D.C., 1993, pp. 425.
96. K. G. Maundrell and co-workers, *Yeast* **10**, 1075 (1994).
97. A. Zurlinden and M. E. Schweingruber, *J. Bacteriol.* **176**, 6631 (1994).
98. A. Iwashima and co-workers, *J. Bacteriol.* **172**, 6145 (1990); *ibid.*, **174**, 4701 (1992).
99. A. Schweingruber and co-workers, *Genetics* **130**, 445 (1992).

DAVID BURDICK
Hoffmann-La Roche Inc.

Related Articles

Vitamins, Survey; Feeds and feed additives